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Enhanced Antioxidative Effect of Ovalbumin Due to Covalent Binding of Polysaccharides

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The antioxidant action of ovalbumin was remarkably enhanced by covalent binding of dextran or galactomannan through a controlled Maillard reaction. A significant improvement in the emulsifying properties of ovalbumin was also observed in the ovalbumin–polysaccharide conjugates. The resulting high affinity to oil suggested that the radical scavenging activity of ovalbumin may be elevated by covering oil surfaces with the ovalbumin–polysaccharide conjugate. The emulsifying activity and emulsion stability of the ovalbumin–polysaccharide conjugates were not affected in acidic pH or high salt conditions, while commercial emulsifiers were greatly affected in these conditions. The conjugates of ovalbumin with dextran or galactomannan can be used as a bifunctional food additive having excellent antioxidant effect and emulsifying properties.

INTRODUCTION

Rancidification causes not only reduction of the shelf life and nutritional value of food products but also toxicity on eating. It is well-known that the progress of lipid oxidation can be prevented by adding antioxidants to the food product or packaging for food product in an anaerobic condition. Although synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have widely been used for the processing of foods, they have been suspected to be responsible for liver damage and carcinogenesis in laboratory animals (Witchi, 1986; Grice, 1988). On the other hand, many studies on naturally existing nontoxic antioxidants have been carried out with great effort. Ovalbumin, which has a protective effect on lipid oxidation (Goto and Shibazaki, 1971), is one of the promising safe substances among them, although its effect is not so strong as that of BHT or BHA. Thus, our attempts were made to enhance the potential antioxidative activity of ovalbumin.

Currently, suppression of rancidification by forming Maillard reaction products has been demonstrated in model systems (Kirigaya et al., 1969; Kato, 1973; Lingnert and Erikson, 1980a,b) as well as in food products (Lingnert, 1980; Lingnert and Lundgren, 1980; Cho et al., 1988; Tanaka et al., 1990). Our recent work showed that the conjugation of ovalbumin with dextran was formed in a controlled dry-heating through Maillard reaction between the free amino groups in the protein and reducing-end carbonyl group in the polysaccharide, and the ovalbumin–dextran conjugate favorably had excellent emulsifying properties (Kato et al., 1990; Kato and Kobayashi, 1991). Thus, we employed this technique to give more powerful antioxidative activity to ovalbumin, because of the masking effect of the oil surface due to the high affinity to oil and the radical scavenger effect of the conjugate. This paper describes enhanced antioxidative properties of ovalbumin due to covalent linking with dextran or mannase hydrolysate of guar gum (galactomannan).

MATERIALS AND METHODS

Materials. Dextran (molecular weight 60 000–90 000) was purchased from Wako Pure Chemicals Industries Ltd., Osaka. Mannase hydrolysate of guar gum (galactomannan, the molecular weight of the main component was 15 000) was supplied from Taiyo Chemicals Co. Nicotinamide adenine dinucleotide (NADPH), nitroblue tetrazolium (NBT), and phenazine methosulfate (PMS) were purchased from Sigma Chemical Co. Celite and methyl linoleate were from Wako. Thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA) were also from Wako. Other chemicals were all of analytical grade. Salad oil, which consists of soybean and

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rapeseed oil without any additive, was obtained from Nisshin Seiyu Ltd., Tokyo. Distilled water was further purified before use (above 10 MΩ/cm) by a Mill-QII reagent water system (Japan Millipore Ltd.).

Preparation of Ovalbumin Conjugate with Polysaccharide. Ovalbumin was prepared from fresh egg white by the crystallization method in sodium sulfate and recrystallized five times (Kekwick and Cannan, 1936). Galactomannan was dialyzed against deionized water for 2 days at 4 °C. Ovalbumin was dissolved in water and freeze-dried with dextran or galactomannan in weight ratios of 1:5 or 1:1, respectively. Lyophilized mixtures were incubated at 60 °C for 3 weeks under 65% relative humidity as previously described (Kato et al., 1990). The resulting samples were used as ovalbumin–polysaccharide conjugates.

Electrophoresis in SDS–Slab Polyacrylamide Gel. SDS–slab polyacrylamide gel electrophoresis was conducted according to the method of Laemmli (1970) using 10% acrylamide separating gel and 3% stacking gel containing 0.1% SDS. Protein samples (20 μL, 0.2%) were heated at 100 °C for 3 min in Tris–glycine buffer, pH 8.8, containing 1% SDS. Electrophoreses were carried out at constant current of 30 mA for 1.5 h using an electrophoretic buffer of Tris–glycine containing 0.1% SDS. The gel sheets were stained for proteins and carbohydrates with Coomassie blue G-250 and Fuchsin, respectively.

Antioxidative Assay in Model System and Actual Food System. A powder model system (Goto and Shibazaki, 1971) was employed to monitor the antioxidant activities of ovalbumin and conjugates of ovalbumin with polysaccharide. Celite was treated with nitrohydrochloric acid for 1 week and subsequently washed with deionized water until the chlorinity reaction disappeared. The purified Celite used as a food model matrix was mixed in water with ovalbumin samples and freeze-dried in a given weight ratio. The resulting powder was mixed with methyl linoleate dissolved in ethyl ether in the weight ratio 3:1, and the residual solvent was evaporated in a cabinet drier. Four grams of the powder food model was put into a Petri dish with diameter of 9 cm and incubated at 20 °C in the dark without humidity regulation. Lipid oxidation in the model system was monitored by measuring peroxide value (POV) and thiobarbituric acid (TBA) value. POV and TBA values were determined as described by Goto and Shibazaki (1971); the TBA value is expressed as absorbance at 532 nm/g of methyl linoleate. The antioxidant effect in an actual food system was assessed by monitoring the POV of salad oil oxidized by the method described by the Association of Official Analytical Chemists (AOAC method). The test samples were prepared by mixing the protein–polysaccharide conjugates with salad oil in 1% concentration on weight basis.

Determination of Superoxide Scavenging Capacity. The scavenging capacity of superoxides was determined according to the method of Ponti et al. (1978). Superoxides were measured by the increasing amount of developed diformazan at 560 nm for 1 min after the addition of PMS solution to NADPH and NBT solutions under aerobic condition. The reaction is as follows:

$$NADPH + H^+ + PMS \rightarrow NADP^+ + PMSH_2$$

$$PMSH_2 + 2O_2 \rightarrow 2O_2^{\cdot -} + 2H^+ + PMS$$

$$NBT + 2Cl^- + 4O_2^{\cdot -} + 4H^+ \rightarrow \text{diformazan} + 4O_2 + 2HCl$$

Measurement of Emulsifying Properties. The emulsifying properties were determined according to the modified method of Pearce and Kinsella (1978). An emulsion was prepared by homogenization of 1.0 mL of corn oil and 3 mL of a 0.1% sample solution, using an Ultra Turrax (Hansen & Co., West Germany) at 12 000 rpm for 1 min at 20 °C. A 100-μL portion of emulsion was taken from the bottom of the test tube at time 0, 1, 2, 3, 5, and 10 min and diluted with 5 mL of 0.1% sodium dodecyl sulfate solution. The turbidity of the diluted emulsion was then determined at 500 nm. The relative emulsifying activity was represented as the absorbance at 500 nm measured immediately after emulsion formation (0 min). The emulsion stability was estimated by measuring the half-life of the decay of the emulsion, estimated from the turbidity curves of emulsion during standing for 10 min.

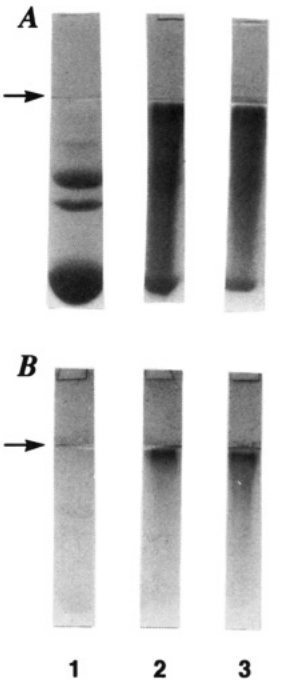

Figure 1. SDS–polyacrylamide gel electrophoresis patterns of ovalbumin–polysaccharide conjugates: (A) protein stain; (B) carbohydorate stain. (Lane 1) Native ovalbumin; (lane 2) ovalbumin–dextran conjugate; (lane 3) ovalbumin–galactomannan conjugate. Arrows indicate the position of the boundary between stacking (upper) and separating (lower) gels.

RESULTS AND DISCUSSION

Figure 1 shows SDS–polyacrylamide gel electrophoretic patterns of ovalbumin and its conjugate with dextran or galactomannan. Covalent attachment of these polysaccharides to ovalbumin was confirmed from the electrophoretic profiles where the conjugates exhibited a broad band for both protein and carbohydrate stains near the boundary between stacking and separating gels (Figure 1, lanes 2 and 3).

Figure 2 shows antioxidative effects of ovalbumin and ovalbumin–dextran conjugate using a powder model system. Ovalbumin–dextran mixture (unheated) and ovalbumin–dextran conjugate (heated for 3 weeks in dry state) were added to the purified Celite powder to give final concentrations of 2.5, 1.25, or 0% (control) for methyl linoleate. The Celite powder containing methyl linoleate was used as a dried food model to estimate lipid oxidation. A significant suppression of lipid oxidation was observed in the tested powders coexisting with the ovalbumin–dextran mixture. With the elongation of incubation time at 20 °C, the antioxidative effect of the ovalbumin–dextran conjugate was more pronounced than that of the ovalbumin–dextran mixture. A similar tendency was observed in the case of the ovalbumin–galactomannan conjugate. As shown in Figure 3, the antioxidative activity of ovalbumin was promoted by covalent binding with galactomannan.

The generation of rancid odors was also suppressed during storage of 1 week in the model food supplemented with ovalbumin–polysaccharide conjugate, while it appeared within 2 and 4 days in the cases of control (without conjugate or mixture) and ovalbumin–polysaccharide mixture, respectively. It appears that both conjugates specifically inhibit the progress of the initial oxidation.

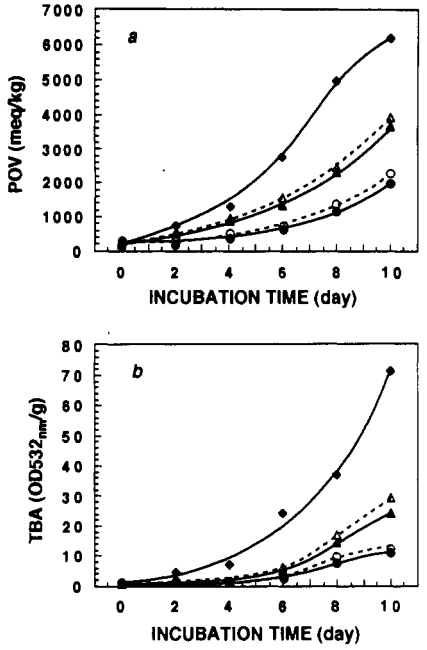

Figure 2. Antioxidative effect of ovalbumin–dextran conjugates in a powder food model system: (a) POV; (b) TBA. (◆) Control (no addition); (△) 1.25% ovalbumin–dextran mixture; (▲) 2.5% ovalbumin–dextran mixture; (○) 1.25% ovalbumin–dextran conjugate; (●) 2.5% ovalbumin–dextran conjugate.

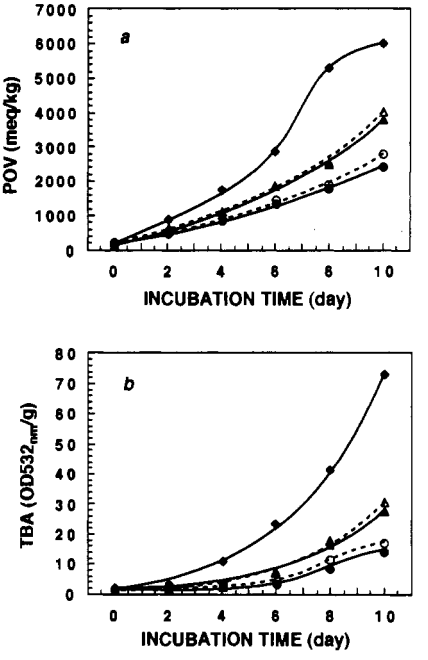

Figure 3. Antioxidative effect of ovalbumin–galactomannan conjugates in a powder food model system: (a) POV; (b) TBA. (◆) Control (no addition); (△) 1.25% ovalbumin–galactomannan mixture; (▲) 2.5% ovalbumin–galactomannan mixture; (○) 1.25% ovalbumin–galactomannan conjugate; (●) 2.5% ovalbumin–galactomannan conjugate.

Table I summarizes the oxidation rate of methyl linoleate in the powder model containing ovalbumin–polysaccharide mixtures or conjugates for 10 days of incubation at 20 °C. The suppression effect of lipid oxidation was increased in proportion to sample concentration. The antioxidation activities of these conjugates were 1.4–1.9 times their mixtures of the same concentration when POV was used as an indicator of oxidation, while the activities of these conjugates were 1.8–2.4 times their mixtures when TBA was used as an indicator. The antioxidative effect of the ovalbumin–dextran conjugate was almost the same as that of the ovalbumin–galactomannan conjugate, although the absolute weight fraction of ovalbumin was much higher in the ovalbumin–galactomannan conjugate (50%) than in the ovalbumin–dextran conjugate (16.7%). This suggests that the antioxidative effect of the conjugates is attributed to either the products of Maillard reaction or the conformational changes in ovalbumin in conjugation.

Table I. Relative Oxidation Rate of Methyl Linoleate in Powder Model System for 10 Days of Incubation at 20 °C

	POV,[a] %	TBA,[a] %
ovalbumin and dextran		
no addition (control)	100	100
mixture (1.25%)	63.3	41.6
mixture (2.5%)	59.1	34.0
conjugate (1.25%)	36.9	17.4
conjugate (2.5%)	31.6	15.7
ovalbumin and galactomannan		
no addition (control)	100	100
mixture (1.25%)	67.1	42.3
mixture (2.5%)	63.6	37.8
conjugate (1.25%)	46.9	23.3
conjugate (2.5%)	40.2	19.5

[a] POV, peroxide value; TBA, thiobarbituric acid value.

Table II. Inhibitory Activity of Superoxide Generation[a] of Ovalbumin–Polysaccharide Conjugates

	inhibitory rate, %
no addition (control)	0.0
native OVA (0.1%)[b]	14.95 ± 1.56[e]
OVA–DX mixture (0.1%)[b]	17.70 ± 5.36
OVA–DX conjugate (0.1%)[b]	25.41 ± 1.39
OVA–GM mixture (0.1%)[b]	17.08 ± 2.63
OVA–GM conjugate (0.1%)[b]	29.22 ± 1.96
BHA (0.001%)[c]	27.79 ± 6.49
BHT (0.001%)[c]	29.45 ± 6.72
SOD (16 units/mL)[d]	86.70 ± 1.02

[a] Superoxide was generated by the NADPH–PMS–NBT reaction system under aerobic conditions. NADPH, nicotinamide dinucleotide phosphate reduced form; PMS, phenazine methosulfate; NBT, nitroblue tetrazolium. [b] Sample concentration was adjusted as protein contents of 0.1%. OVA, ovalbumin; DX, dextran; GM, galactomannan. [c] BHT, butylated hydroxytoluene; BHA, butylated hydroxyanisole. [d] SOD, superoxide dismutase obtained from bovine erythrocyte. [e] Mean ± SD, $n = 4$.

Table II shows the effect of native ovalbumin, ovalbumin–polysaccharide mixtures, and ovalbumin–polysaccharide conjugates on superoxides generated from the NADPH–PMS–NBT reaction system under aerobic condition. The inhibition of superoxides generating from the reaction system was about 15% by addition of 0.1% native ovalbumin, whereas it was about 87% by superoxide dismutase (16 units/mL) from bovine erythrocyte. Addition of 0.1% (protein concentration) ovalbumin–dextran conjugate and ovalbumin–galactomannan conjugate scavenged about 25 and 29% of generating superoxides, respectively. The inhibitory rate of generating superoxides of native ovalbumin was significantly elevated by conjugation with dextran or galactomannan through the controlled Maillard reaction. The superoxide-scavenging activities of 0.1% of these conjugates were almost similar to those of 0.001% BHT and BHA. The storage stability of oil-containing processed foods is mostly dependent on the rancidity of lipids. Lipid peroxidation plays a crucial role in the deterioration of nutrition and the formation of off-flavor and toxic substances. Many studies have been directed toward retarding the development of rancidity in processed foods. Moreover, research for safe antioxidants has been carried out with great efforts. It is clear, therefore, that ovalbumin–polysaccharide conjugate without the use of chemical reagents can be potentially used in processed foods.

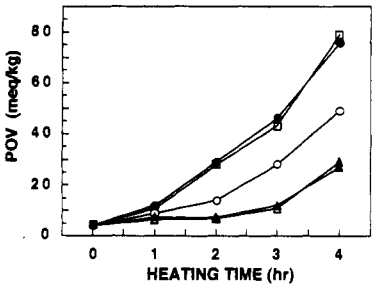

Figure 4. AOM test of protein–polysaccharide conjugates using commercial salad oil. (●) Control (no addition); (○) 1% ovalbumin–dextran conjugate; (□) 1% lysozyme–dextran conjugate; (△) BHT; (▲) BHA.

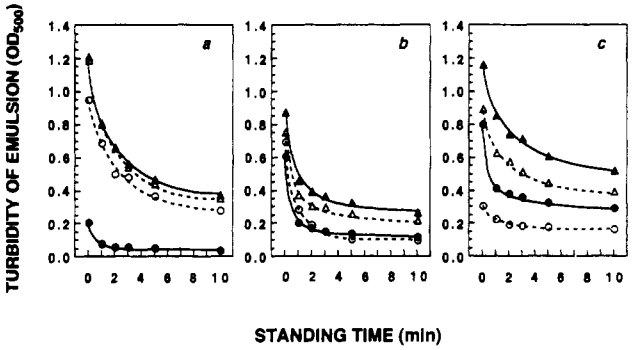

Figure 5. Emulsifying properties of ovalbumin–polysaccharide conjugates in various conditions: (a) in 0.1 M phosphate buffer (pH 7.4); (b) in 0.1 M phosphate buffer (pH 7.4) containing 0.2 M NaCl; (c) in acetate buffer (pH 3.0). (●) Native ovalbumin; (△) ovalbumin–galactomannan conjugate; (▲) ovalbumin–dextran conjugate; (○) commercial emulsifier, sucrose fatty acid ester (HLB11) from Taiyo Kagaku.

An attempt to assess the antioxidative effect of the ovalbumin–polysaccharide conjugates in the actual food system was made by using commercial salad oil. Figure 4 shows the changes in the peroxide value of salad oil oxidized according to the AOAC method in the presence of ovalbumin–dextran conjugate. For the control substance, lysozyme–dextran conjugate (Nakamura et al., 1991) was used. BHT and BHA were also used as the standard substance. Ovalbumin–dextran conjugate inhibited significantly salad oil oxidation, although the effect was not so strong as those in the presence of 0.05% BHA or BHT. On the other hand, the suppression effect of active oil oxidation was not observed in the lysozyme–dextran conjugate. This observation suggests that a potential radical scavenging activity of ovalbumin was attributed to the antioxidative effect of ovalbumin–polysaccharide conjugate. It is well-known that ovalbumin contains a high amount of sulfur-containing amino acid. Ovalbumin contains 1 disulfide bond, 4 reactive sulfhydryl groups, and 15 methionine residues per molecule. The antioxidative effect of the ovalbumin–polysaccharide conjugate may be due to the effective exposure of active sulfhydryl groups in the ovalbumin molecule by the conjugation with polysaccharide.

Figure 5 shows the emulsifying properties of ovalbumin and ovalbumin–polysaccharide conjugates. Conjugation of ovalbumin with polysaccharide showed much better emulsifying properties than native ovalbumin. Only a slight difference was observed between the ovalbumin–dextran conjugate and the ovalbumin–galactomannan conjugate. The emulsifying properties of these conjugates were better than those of a commercial emulsifier, sucrose fatty acid ester in the presence of salt (Figure 5b) and in acidic pH (Figure 5c). The relative emulsifying activity and emulsion stability were calculated from the curves in Figure 5. As shown in Table III, the relative emulsifying activities of the ovalbumin–dextran and ovalbumin–galactomannan conjugates in 0.1 M phosphate buffer, pH 7.4, were about 10 and 15 times that of native ovalbumin, respectively. In parallel, the emulsion stability of these conjugates was also better than that of native ovalbumin. Moreover, the excellent emulsifying properties of these conjugates were maintained even in the presence of 0.2 M NaCl or in acidic pH, while the commercial emulsifier was greatly affected under these conditions. This observation suggests that the affinity of ovalbumin to oil was remarkably improved by covalent linking with polysaccharides. The resulting high affinity to oil may elevate the radical scavenging activity of ovalbumin, because the oil surfaces were closely covered with the ovalbumin–polysaccharide conjugate.

Table III. Relative Emulsifying Activity and Emulsion Stability of Native Ovalbumin, Ovalbumin–Dextran Conjugate, and Ovalbumin–Galactomannan Conjugate in the Various Conditions

	pH 7.4[a]	0.2 M NaCl[b]	pH 3.0[c]
relative emulsifying activity[d]			
native OVA	0.202	0.604	0.802
OVA–DX conjugate	1.205	0.875	1.160
OVA–GM conjugate	1.186	0.750	0.897
commercial emulsifier[e]	0.944	0.693	0.304
emulsion stability[f]			
native OVA	0.6	0.6	1.2
OVA–DX conjugate	2.6	1.4	5.0
OVA–GM conjugate	2.5	1.1	4.8
commercial emulsifier	2.6	0.8	5.0

[a] pH 7.4, 1/15 M sodium phosphate buffer. [b] 0.2 M NaCl, 1/15 M sodium phosphate buffer (pH 7.4) containing 0.2 M NaCl. [c] pH 3.0, 1/15 M sodium citrate buffer. [d] Absorbance at 500 nm measured immediately after emulsion formation (0 min). [e] Sucrose fatty acid ester (HLB11) from Taiyo Kagaku. [f] Half-life time (min) of the decay of emulsion. OVA, ovalbumin; DX, dextran; GM, galactomannan.

Table IV. Food Safety of Ovalbumin–Polysaccharide Conjugate

		bacterial mutagenesis test	
	LD_{50}[a]	Ames test[b]	*rec* assay[c]
ovalbumin–dextran conjugate	>7.5	negative	negative
ovalbumin–galactomannan conjugate	>7.5	negative	negative

[a] Lethality of these conjugates was tested by oral administration to mice, and the value represents an amount of compound that causes 50% death of tested mice (g/kg). [b] According to the method of Maron and Ames (1983). [c] According to the method of Kada et al. (1972).

Animal dose test and bacterial mutagenesis test were done to confirm the food safety of the ovalbumin–dextran and ovalbumin–galactomannan conjugates. As shown in Table IV, these ovalbumin–polysaccharide conjugates are nontoxic for oral administration to mice, and they are negative for the Ames test and the *rec* assay. It is suggested from these results that these substances are safe and suitable for industrial applications, because of its preparation without chemical reagents.

Thus, we successfully developed a natural macromolecular antioxidant having good emulsifying properties. The development of the protein emulsifier having antioxidative properties may be of great benefit for food industries because food emulsions are susceptible to rancidity through lipid oxidation.

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